S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit

Journal:	*Physiologia Plantarum*
Manuscript ID	PPL-2016-00373
Manuscript Type:	Regular manuscript - Ecophysiology, stress and adaptation
Date Submitted by the Author:	03-Nov-2016
Complete List of Authors:	Silveira, Neidiquele; Agronomic Institute, Center of Ecophysiology and Biophysics Marcos, Fernanda; University of Campinas, Department of Plant Biology, Institute of Biology Frungillo, Lucas; University of Edinburgh, School of Biological Sciences, Institute of Molecular Plant Sciences Moura, Bárbara; University of Campinas, Department of Plant Biology, Institute of Biology Seabra, Amedea; Federal University of ABC, Center of Natural and Human Sciences Salgado, Ione; University of Campinas, Department of Plant Biology, Institute of Biology Machado, Eduardo; Agronomic Institute, Center of Ecophysiology and Biophysics Hancock, John; University of the West of England, School of Biosciences Ribeiro, Rafael; University of Campinas, Department of Plant Biology, Institute of Biology
Key Words:	Antioxidant enzymes, Drought, Photosynthesis, Nitric oxide, Saccharum

SCHOLARONE™
Manuscripts

_S_-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit

Neidiquele M. Silveira[a], Fernanda C. C. Marcos[b], Lucas Frungillo[c], Bárbara B. Moura[b], Amedea B. Seabra[d], Ione Salgado[b], Eduardo C. Machado[a], John T. Hancock[e] and Rafael V. Ribeiro[b,*]

[a]Laboratory of Plant Physiology "Coaracy M. Franco", Center R&D in Ecophysiology and Biophysics, Agronomic Institute (IAC), Campinas SP, Brazil

[b]Department of Plant Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas SP, Brazil

[c]School of Biological Sciences, Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh, UK

[d]Center of Natural and Human Sciences, Federal University of ABC, Santo André, SP, Brazil

[e]Centre for Research in Plant Science, University of the West of England (UWE), Bristol, UK

*Corresponding author, e-mail: rvr@unicamp.br

For Peer Review

Abbreviations:

A, leaf CO_2 assimilation; ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; C_i, intercellular CO_2 concentration; DAB, 3,3´diaminobenzidine; ETR, apparent electron transport rate; g_S, stomatal conductance; GSH, glutathione; GSNO, *S*-nitrosoglutathione; MDA, malondialdehyde; NO, nitric oxide; $O_2^{\cdot -}$, superoxide anion; PEG, polyethylene glycol; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PPFD, photosynthetic photon flux density; PSII, photosystem II; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC, relative water content; SOD, superoxide dismutase; WD, water deficit.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

31 **Abstract**

32

33 Water deficit is a major environmental constraint on crop productivity and performance and
34 nitric oxide (NO) is an important signaling molecule associated with many biochemical and
35 physiological processes in plants under stressful conditions. This study aims to test the
36 hypothesis that leaf spraying of *S*-nitrosoglutathione (GSNO), a NO donor, improves the
37 antioxidant defense in both roots and leaves of sugarcane plants under water deficit, with
38 positive consequences for photosynthesis. In addition, the role of key photosynthetic
39 enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and
40 phosphoenolpyruvate carboxylase (PEPC) in maintaining CO_2 assimilation of GSNO-
41 sprayed plants under water deficit were evaluated. Sugarcane plants were sprayed with
42 water or GSNO 100 μM and subjected to water deficit, by adding polyethylene glycol
43 (PEG-8000) to the nutrient solution. Sugarcane plants supplied with GSNO presented
44 increases in the activity of antioxidant enzymes such as SOD in leaves and CAT in roots,
45 indicating higher antioxidant capacity under water deficit. Such adjustments induced by
46 GSNO were sufficient to prevent oxidative damage in both organs and were associated with
47 better leaf water status. As consequences, GSNO spraying alleviated the negative impact of
48 water deficit on stomatal conductance and photosynthetic rates, with plants also showing
49 increases in Rubisco activity under water deficit.

50

51 **Keywords:** Antioxidant enzymes; Drought; Photosynthesis; Nitric oxide; *Saccharum*.

52

53

54

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

55 **Introduction**

56

57 Low water availability is the main abiotic stress affecting sugarcane metabolism and
58 reducing crop yield and biomass production (Ribeiro et al. 2013, Sales et al. 2013).
59 Sugarcane plants facing water deficit present reductions in CO_2 assimilation, transpiration,
60 stomatal opening, and decreases in tillering and culm length (Machado et al. 2009, Silva et
61 al. 2012). While stomatal closure reduces CO_2 diffusion into mesophyll and then limit
62 sugarcane photosynthesis under mild water deficit, metabolic limitation of photosynthesis
63 occurs under severe water deficit through decreases in the activity of key photosynthetic
64 enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and
65 phosphoenolpyruvate carboxylase (PEPC) (Ghannoum 2009, Lopes et al. 2011). These
66 responses may be associated with impairment of ATP and NADPH synthesis, reducing
67 regeneration of both ribulose-1,5-bisphosphate (RuBP) and phosphoenolpyruvate (PEP) in
68 C_4 plants (Lawlor 2002). In fact, photochemical impairment leading to low ATP and
69 NADPH synthesis is found under drought and it is a result of reduced electron transport
70 rate between PSII and PSI and damage to thylakoid membranes (Lawlor and Cornic 2002).
71 The redox status of cells is also changed under water deficit due to reduced
72 consumption ATP and NADPH in reactions of CO_2 assimilation. Thus, the water deficit
73 can disrupt cellular homeostasis and increase the production of reactive oxygen species
74 (ROS). Increase in superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) concentrations in
75 plant cells is one of the first symptoms of exposure to drought, often accompanied by lipid
76 peroxidation in cell membranes (Yamamoto et al. 2002, Tian and Lei 2006). To prevent or
77 reduce cell damage due to ROS, plants have an antioxidant system based on enzymatic and
78 non-enzymatic reactions. While the non-enzymatic reactions are mediated by ascorbate,

For Peer Review

glutathione, flavonoids, carotenoids and tocopherols, the enzymatic mechanism consists of several enzymes located in different cellular compartments such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT). During cell detoxification, $O_2^{\cdot -}$ produced in the mitochondria, chloroplasts and peroxisomes is dismuted to H_2O_2 by SOD, which is rapidly eliminated by the CAT and APX, producing H_2O and O_2 (Foyer and Noctor 2005, Wu et al. 2012, Huseynova 2012).

Some studies have reported nitric oxide (NO) action in plants under drought (Santisree et al. 2015, Farnese et al. 2015), with this molecule cooperating in ABA-induced stomatal closure (Neill et al. 2003) and also increasing drought tolerance in *Vicia faba* (Garcia-Mata and Lamattina 2001). In fact, NO is an important plant messenger mediating various physiological and biochemical processes, which can directly alter protein structure and activity (Cai et al. 2015). NO has a multifunctional role and its effects on plants can be beneficial or not, depending on its concentration and type of donor when supplied. Recently, we found increases in root growth and maintenance of leaf water status in sugarcane plants under water deficit and supplied with NO. GSNO-sprayed plants exhibited increases in photosynthesis under water deficit, which was a consequence of high stomatal conductance and increased apparent carboxylation efficiency (Silveira et al. 2016).

While high *S*-nitrosothiols content in GSNO-sprayed sugarcane plants suggests a long-term role of NO-mediated responses to water deficit (Silveira et al. 2016), it is unclear whether improved photosynthesis is related to the activity of key photosynthetic enzymes Rubisco and PEPC. Few studies have addressed the effects of NO on Rubisco and PEPC activities, which are likely dependent on plant species and NO donor. For instance, Kovacs (2013) showed that NO can *S*-nitrosated Rubisco in a dose-dependent manner, reducing its activity.

For Peer Review

1
2
3 103 Alternatively, improved performance of NO-supplied plants under drought may be
4
5 104 due to reduced oxidative damage. NO can prevent oxidative damage by activation of
6
7 105 antioxidant mechanisms and maintenance of ROS homeostasis in two turfgrass species
8
9 106 (Hatamzadeh et al. 2014), increasing ascorbate levels and activity of antioxidant enzymes
10
11 107 (Zhang et al. 2008). Furthermore, $O_2^{\cdot -}$ can react with NO to form peroxynitrite (ONOO-)
12
13 108 and therefore control ROS accumulation (Radi et al. 2002). The protective effect of
14
15 109 exogenous application of NO donor molecules has been attributed to the elimination of $O_2^{\cdot -}$
16
17 110 and to increases in the activities of SOD, CAT and APX (Zhao et al. 2008). However, the
18
19 111 consequences of such metabolic changes on photosynthetic metabolism remain unexplored.
20
21 112 The activity of NADPH oxidase is also an important source of ROS under abiotic stress
22
23 113 (Marino et al. 2012), with its activity reduced through *S*-nitrosylation mediated by NO
24
25 114 (Yun et al. 2011).
26
27 115 While there is ample evidence of the involvement of NO in plant responses to
28
29 116 drought, our knowledge about the underlying physiological processes leading to improved
30
31 117 performance is still limited. In such context and using GSNO as a donor of NO, we
32
33 118 hypothesized that leaf GSNO spraying improves the antioxidant defense of sugarcane
34
35 119 plants under water deficit, with positive consequences for photosynthesis during and after
36
37 120 such stressful condition. In addition, the role of key photosynthetic enzymes Rubisco and
38
39 121 PEPC in maintaining CO_2 assimilation in GSNO-sprayed plants under water deficit was
40
41 122 evaluated.
42
43 123
44
45 124
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Material and methods

Plant material and experimental conditions

Sugarcane plants (*Saccharum* spp.) cv. IACSP94-2094 developed by ProCana Program (Agronomic Institute, IAC, Brazil) were propagated by placing mini-stalks from adult plants in trays containing commercial substrate composed of *Sphagnum*, rice straw and perlite in 7:2:1 ratio (Carolina Soil of Brazil, Vera Cruz RS, Brazil). Three-week-old plants with two to three leaves were transferred to modified Sarruge (1975) nutrient solution (15 mmol L^{-1} N (7% as NH_4^+); 4.8 mmol L^{-1} K; 5.0 mmol L^{-1} Ca; 2.0 mmol L^{-1} Mg; 1.0 mmol L^{-1} P; 1.2 mmol L^{-1} S; 28.0 μmol L^{-1} B; 54.0 μmol L^{-1} Fe; 5.5 μmol L^{-1} Mn; 2.1 μmol L^{-1} Zn; 1.1 μmol L^{-1} Cu and 0.01 μmol L^{-1} Mo) and maintained hydroponically in a growth chamber (PGR15, Conviron, Winnipeg MB, Canada) at 30/20 ^{o}C (day/night), 80% relative humidity, 12 h photoperiod (7:00 to 19:00 h) and PPFD of 800 μmol m^{-2} s^{-1}. This PPFD was the maximum intensity at plant level, inside growth chamber. Plants were grown under the above conditions for 30 days prior to water deficit and GSNO spraying.

Sugarcane plants were subjected to water deficit (WD) by adding polyethylene glycol (Carbowax^{TM} PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential as follows: -0.25 MPa on the first day; -0.5 MPa on the second day; -0.6 MPa on the third day; -0.75 MPa on the 6th day of treatment. From this point, the osmotic potential of the nutrient solution was monitored daily and corrected when necessary. After six days under water deficit (-0.75 MPa), plants were transferred to the original nutrient solution (-0.15 MPa) for rehydration for three days.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

149 Sugarcane leaves were sprayed twice a day with freshly prepared GSNO solutions at 150 100 µM. This concentration was based on a previous study in which significant increase of 151 photosynthesis was found in sugarcane plants under water deficit (Silveira et al. 2016). 152 Leaves were sprayed as follows: when the osmotic potential of nutrient solution reached - 153 0.25, -0.5 and -0.6 MPa. The last GSNO spraying was done three days after reaching -0.75 154 MPa (9th day). GSNO spraying was done outside the growth chamber to avoid undesirable 155 interference with other treatments. Plants were then subjected to the following treatments: 156 control, under nutrient solution with osmotic potential of -0.15 MPa + water spray; water 157 deficit (WD), under nutrient solution with osmotic potential of -0.75 MPa + water spray; 158 and WD + GSNO spray (WDG).

159 Sampling was done at the maximum water deficit (12th day after adding PEG-8000 to 160 the nutrient solution) and also at the recovery period (3rd day after returning plants to the 161 control nutrient solution). Samples of leaves and roots were collected, immediately 162 immersed in liquid nitrogen and then stored at -80° C for further enzymatic analyses.

163

164 **Synthesis of *S*-nitrosoglutathione (GSNO)**

165

166 *S*-nitrosoglutathione (GSNO) is an important donor of NO, which is a *S*-nitrosated 167 derivative of the most abundant cellular thiol, glutathione (GSH). GSNO has been 168 considered an internal source of NO and as an essential component of NO-dependent signal 169 transduction pathway (Broniowska et al. 2013). GSNO was synthesized and characterized 170 as previously described (Silveira et al. 2016). Briefly, the reduced glutathione (GSH) was 171 reacted with equimolar sodium nitrite in acidified aqueous solution, acetone was added, and

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

172 the solution was washed with cold water, filtered and the obtained GSNO was freeze-dried
173 for 24 h.
174

175 **Leaf gas exchange and photochemical activity**

176

177 Gas exchange of the first fully expanded leaf with visible ligule was measured daily
178 using an infrared gas analyzer (Li-6400, Licor, Lincoln NE, USA) attached to a modulated
179 fluorometer (6400-40 LCF, Licor, Lincoln NE, USA). Leaf CO_2 assimilation (A), stomatal
180 conductance (g_s) and intercellular CO_2 concentration (C_i) were measured under PPFD of
181 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 400 μmol mol^{-1}. The measurements were
182 performed between 11:00 and 13:00 h, as done by Silveira et al. (2016). The vapor pressure
183 difference between leaf and air (VPDL) was 2.2±0.3 kPa and leaf temperature was 29±1 ^oC
184 during the evaluations.

185 Chlorophyll fluorescence was evaluated simultaneously to the leaf gas exchange and
186 the apparent electron transport rate (ETR) was estimated as ETR= ϕ_{PSII} ×PPFD×0.85×0.4,
187 in which ϕ_{PSII} is the effective quantum efficiency of photosystem II (PSII), 0.85 is the light
188 absorption and 0.4 is the fraction of light energy partitioned to PSII (Edwards and Baker
189 1993, Baker 2008). The non-photochemical quenching was calculated as NPQ=(F_M −
190 F_M')/F_M'. The relative energy excess at PSII level was calculated as EXC = [(F_V/F_M) −
191 ϕ_{PSII}]/(F_V/F_M), in which F_V/F_M is the potential quantum efficiency of photosystem II
192 (Bilger et al. 1995). A chlorophyllmeter (CFL 1030, Falker, Porto Alegre RS, Brazil) was
193 used to evaluate chlorophyll *a* and *b* and the relative content of chlorophyll (Chl) was
194 calculated as chlorophyll *a* + *b*.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

195 Measurements were taken at the beginning of the experiment (before reducing the
196 osmotic potential of nutrient solution), when the osmotic potential of nutrient solution
197 reached -0.75 MPa, at the maximum water deficit, and three days after returning plants to
198 the control condition (recovery period).
199
200 **Relative water content**
201
202 The relative water content (RWC) was calculated in leaf discs according to Jamaux et
203 al. (1997): RWC=100*[(FW-DW)/(TW-DW)], in which FW, TW and DW are the fresh,
204 turgid and dry weights, respectively.
205
206 **Activity of photosynthetic enzymes**
207
208 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39)
209
210 Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of
211 Bicine-NaOH buffer 100 mM (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM
212 $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10
213 μM leupeptin. The resulting solution was centrifuged at 14000 *g* for 5 min at 4 °C. The
214 initial activity of Rubisco was measured using as medium 100 mM bicine-NaOH (pH 8.0)
215 containing 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25
216 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-
217 phosphoglyceric phosphokinase and 80 nkat creatine phosphokinase, incubated at 25 °C.
218 The oxidation of NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

219 (RuBP). A similar aliquot was incubated with the reaction medium for 10 min at 25 °C and
220 total Rubisco activity was measured after adding RuBP. The reduction of absorbance at 340
221 nm was monitored for 3 min. The activation state of Rubisco was calculated as the ratio
222 between the initial and total activities (Sage et al. 1988, Reid et al. 1997).

223

224 Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31)

225

226 Approximately 100 mg of leaves were macerated and homogenized with 0.5 mL of
227 100 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA and 1 mM PMSF and
228 centrifuged at 14000 *g* for 25 min at 4 °C and the supernatant collected. The reaction
229 medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5
230 mM glucose 6-phosphate, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase and 0.3 mM
231 NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate and incubated at
232 30 °C. The oxidation of NADH was monitored for 1 min (Degl'innocenti et al. 2002).

233

234 **Reactive oxygen species**

235

236 The quantification of hydrogen peroxide (H_2O_2) in plant material was performed
237 following the method of Alexieva et al. (2001). Homogenates were obtained from 0.1 g of
238 fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP)
239 and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at
240 10000 *g* and 4 ^{o}C for 15 min. The reaction medium consisted of 1.2 mL of KI 1 mM,
241 potassium phosphate buffer (pH 7.5 and 0.1 M) and crude extract. The microtubes were
242 incubated on ice under dark for 1 h. After this period, the absorbance was read at 390 nm.

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

243 A standard curve was obtained with H_2O_2 and the results were expressed as µmol H_2O_2 g^{-1}
244 FW.

245 To determine the concentration of the superoxide anion ($O_2^{\cdot-}$), 50 mg of samples were
246 incubated in an extraction medium consisting of 100 µM EDTA, 20 µM NADH, and 20
247 mM sodium phosphate buffer, pH 7.8 (Kuo and Kao 2003). The reaction was initiated by
248 adding 100 µL of 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28 °C
249 under stirring for 5 min and the absorbance was read at 480 nm for 5 min. Superoxide anion
250 production was assessed by the accumulation of adrenochrome using a molar absorption
251 coefficient of 4.0×10^3 M^{-1} (Gay and Gebicki 2000).
252

253 **Lipid peroxidation**

254

255 The concentration of malondialdehyde (MDA) was measured and used as a proxy of
256 lipid peroxidation. One hundred fifty mg of plant tissue were macerated in 2 mL of 0.1%
257 TCA (w/v) and centrifuged at 10000 *g* for 15 min. The supernatant was added to 1.0 mL of
258 0.5 % thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture was incubated at 95 °C.
259 Two hours after the reaction, the absorbance was measured at 532 and 600 nm. The
260 absorbance at 600 nm was subtracted from the absorbance at 532 nm and the MDA
261 concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and
262 Packer 1968).

263

264 **Protein extraction and antioxidant activity**

265

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

266 Plant extracts were obtained from the macerate of 0.1 g of fresh tissue (roots or 267 leaves) with liquid nitrogen, 1% PVPP and 2 mL of extraction medium containing 0.1 M 268 potassium phosphate buffer (pH 6.8), 0.1 mM EDTA and 1 mM PMSF. After 269 centrifugation of the homogenates at 15000 *g* for 15 min and 4 ^{o}C, supernatants (crude 270 extracts) were collected and preserved on ice. The protein levels of the enzymatic extracts 271 were determined by the Bradford method (Bradford 1976) using bovine serum albumin 272 (BSA) as the standard.

273 Catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure 274 described in Havir and McHale (1987). The reaction medium consisted of 3 mL of 100 mM 275 potassium phosphate buffer (pH 6.8), deionized water, 125 mM H_2O_2 and crude extract. 276 The reaction was carried out in a water bath at 25 ^{o}C for 2 min and activity assessed by the 277 decrease in absorbance at 240 nm. The CAT activity was calculated using the molar 278 extinction coefficient of 36 M^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} of protein.

279 Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by 280 Nakano and Asada (1981). The reaction medium was composed by 3 mL of 100 mM 281 potassium phosphate buffer (pH 6.0), deionized water, 10 mM ascorbic acid, 10 mM H_2O_2 282 and crude extract. The reaction was carried out in a water bath at 25 ^{o}C for 2 min and 283 activity monitored by the decrease in absorbance at 290 nm, using the molar extinction 284 coefficient of 2.8 M^{-1} cm^{-1} to measure the APX activity, which was expressed as µmol 285 min^{-1} mg^{-1} of protein.

286 Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to 287 Giannopolitis and Ries (1977). The reaction medium consisted of 3 mL of 100 mM sodium 288 phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, crude 289 extract, 100 µM riboflavin and 1 mM nitro blue tetrazolium chloride (NBT). A group of

For Peer Review

1
2
3 290 tubes was exposed to light (fluorescent lamp of 30 W) for 8 min, and another group
4
5 291 remained in darkness. The absorbance was measured at 560 nm and one unit of SOD is the
6
7
8 292 amount of enzyme required to inhibit the NBT photoreduction in 50%, being expressed as
9
10 293 U min^{-1} mg^{-1} of protein.
11
12
13 294
14
15 295 **Superoxide dismutase zymogram**
16
17 296
18
19
20 297 In order to check the cellular compartment in which the first line of enzymatic
21
22 298 defense against oxidative damage was active, the identification of SOD isoforms was
23
24 299 performed by native-PAGE electrophoresis in polyacrylamide gel (12.5%) at 4 ^oC under
25
26
27 300 constant current of 40 mA in Tris-HCl (pH 8.3) and glycine buffer. The protein extract was
28
29 301 obtained after maceration of a composite leaf sample (0.4 g, composed by ~0.1 g per
30
31
32 302 replicate) (Ferreira et al. 2002). SOD isoforms were identified through specific inhibition
33
34 303 by H_2O_2 and KCN, with isoforms being classified as Mn-SOD (resistant to both inhibitors),
35
36
37 304 Fe-SOD (resistant only to KCN) and Cu/Zn-SOD (inhibited by both inhibitors) (Azevedo et
38
39 305 al. 1998).
40
41
42 306
43
44 307 **Histochemical analysis**
45
46
47 308
48
49 309 Samples of three leaves per plant (1 cm^2) in three plants per treatment were cut and
50
51 310 immediately immersed in a solution of 1 mg mL^{-1} of DAB-HCl, adjusted to pH 5.6 with
52
53
54 311 NaOH and incubated in dark for 8 h. Samples were taken at the maximum water deficit and
55
56 312 they were cleared in 96% ethanol (Faoro and Iriti 2005) before transversal hand cuts.
57
58
59 313 Samples were examined with a light microscope Olympus BX41 (Tokyo, Japan) fitted with
60

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

314 a digital camera (Media Cybernetics PL-A624, Bethesda MD, USA). H_2O_2 was visualized
315 as a reddish-brown coloration. As a negative control, DAB solution was supplemented with
316 10 mM ascorbic acid (Faoro et al. 2001).
317

318 **Data analysis**

319

320 The experimental design was completely randomized and data were subjected to the
321 ANOVA procedure. The Scott-Knott test was used to compare treatments when
322 significance was found ($P<0.05$). The results presented are the mean ± SD and the number
323 of replicates is stated in each figure caption.
324

325 **Results**

326

327 **GSNO improves leaf water status and chlorophyll content under water deficit**

328

329 As compared to well-hydrated plants, water deficit caused significant reduction in
330 relative water content (RWC) of sugarcane plants at the maximum water deficit. Such
331 reduction in RWC was alleviated when plants were sprayed with GSNO (Fig. 1A). Low
332 water availability also reduced the chlorophyll content of sugarcane plants at the maximum
333 water deficit and recovery period. Again, GSNO spraying alleviated the decreases in
334 chlorophyll content caused by water deficit (Fig. 1B).
335

336 **GSNO improves leaf gas exchange, photochemistry and increases Rubisco activity**
337 **under water deficit**

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

338

339 The water deficit induced a large reduction in leaf CO_2 assimilation of sugarcane
340 plants from the 4th day of treatment, with GSNO treatment improving leaf CO_2 assimilation
341 during the water deficit (Fig. 2A). The stomatal conductance was affected by water deficit
342 and GSNO supplying in a similar way as compared to leaf CO_2 assimilation (Fig. 2B).
343 There was a significant increase in intercellular CO_2 concentration (C_i) at the maximum
344 water deficit when plants were not supplied with GSNO and subjected to water deficit (Fig.
345 2C). The apparent electron transport rate (ETR) was reduced due to water deficit. However,
346 such negative effect of water deficit was nulled by GSNO only at the beginning of water
347 deficit treatment (Fig. 2D). The consumption of electrons per CO_2 assimilated (ETR/A) was
348 increased in plants under water deficit conditions. However, plants sprayed with GSNO
349 presented lower ETR/A than plants under low water availability and without GSNO
350 supplying, mainly at the maximum water deficit (Fig. 2E). Leaf GSNO spraying caused the
351 lowest non-photochemical quenching at the maximum water deficit (Fig. 2F). While water
352 deficit caused increases in excessive light energy at PSII (EXC), GSNO spraying led to
353 reductions in EXC and then control plants and those treated with GSNO presented similar
354 values during the first days of water deficit (data not shown).

355 Rubisco activity was decreased by 65% in plants under water deficit and increased
356 above 82% from controls by GSNO supplying (Fig. 3A). On average, GSNO-supplied
357 plants presented Rubisco activity almost two-fold higher than control ones at the maximum
358 water deficit (Fig. 3A). On the other hand, the Rubisco activation state (Fig. 3A, insert) and
359 PEPC activity were not affected by water deficit or GSNO supplying (Fig. 3B).

360

361 **GSNO and oxidative damage**

For Peer Review

Even without a statistical difference in leaf H_2O_2 levels among treatments (data not shown), the histochemical analysis revealed H_2O_2 close to the stomata of plants under water deficit and without GSNO supplying (Fig. 4). At the maximum water deficit and recovery period, the highest $O_2^{\cdot-}$ concentrations in both leaves and roots were found in plants subjected to water deficit. Interestingly, plants supplied with GSNO and control ones presented similar leaf and root $O_2^{\cdot-}$ concentrations (Fig. 5A,B). Leaf lipid peroxidation was not changed by treatments. However, there was an increase in root MDA content of plants under water deficit without GSNO supplying at the recovery period (Fig. 5C,D).

GSNO stimulates the antioxidant system under water deficit

In plants sprayed with GSNO, root CAT activity increased by 44% at the maximum water deficit and by 49% at the recovery period, as compared to control ones (Fig. 6B). Leaf CAT activity decreased due to water deficit, regardless whether GSNO was supplied or with evaluation time (Fig. 6A). At the maximum water deficit, leaf APX activity was increased only in plants subjected to water deficit and without GSNO treatment (Fig. 6C). In roots, APX activity decreased due to water deficit and GSNO spraying did not modify this response at the maximum water deficit and recovery period (Fig. 6D). Compared to the control condition, leaf SOD activity was significantly increased by GSNO spraying at the maximum water deficit (Fig. 6E). Root SOD activity was increased by water deficit, regardless GSNO spraying and evaluation time (Fig. 6F).

The SOD zymogram revealed details about the activities of SOD isoforms, which were numbered arbitrarily based on the order of appearance on the gels. When considering

For Peer Review

1
2
3 386 leaf SOD isoforms, five isoforms were identified in control treatment and seven in water
4
5 387 deficit, regardless of GSNO spraying (Fig. 7). There were four and seven root SOD
6
7 388 isoforms in control and water deficit conditions, respectively. In leaves, Cu/Zn-SOD
8
9 389 isoforms *iii* and *iv* were detected only under water deficit, regardless of GSNO spraying.
10
11 390 However, GSNO increased the activity of all SOD isoforms when compared to the other
12
13 391 treatments. In roots, Mn-SOD isoform *iii* and Cu/Zn-SOD isoforms *v* and *vi* were detected
14
15 392 only in plants under water deficit, regardless of GSNO supplying (Fig. 7).
16
17 393
18
19 394 **Discussion**
20
21 395
22
23 396 **How does GSNO improve photosynthesis under water deficit?**
24
25 397
26
27 398 Our findings clearly show that leaf GSNO spraying improves sugarcane tolerance to
28
29 399 water deficit by improving photosynthetic rates. Such improvement in leaf CO_2
30
31 400 assimilation was associated, in part, with higher RWC and higher stomatal conductance
32
33 401 under water deficit (Figs. 1A, 2B). For instance, GSNO treatment increased by seven times
34
35 402 the stomatal conductance at the maximum water deficit as compared to plants sprayed with
36
37 403 water (Fig. 2B). It could be argued that improvements in leaf water status were concomitant
38
39 404 with the stomatal conductance in GSNO-sprayed plants under water deficit, which was
40
41 405 lower than in control plants (Fig. 2B). As consequence, GSNO-sprayed plants would
42
43 406 minimize water vapor loss through transpiration (data not shown) and maximize CO_2
44
45 407 availability in intercellular spaces. Accordingly, control plants and those sprayed with
46
47 408 GSNO presented similar C_i values throughout the experimental period (Fig. 2C).
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

409 Besides the diffusive limitation of photosynthesis imposed by stomatal closure under
410 water deficit, plants may face biochemical limitation under severe drought. Then, GSNO
411 would also improve CO_2 uptake under water deficit through changes in the activity of key
412 photosynthetic enzymes. In fact, plants supplied with GSNO exhibited an increase in
413 Rubisco activity under water deficit (Fig. 3A). Beligni and Lamattina (2002) found that the
414 NO delayed Rubisco mRNA loss caused by treatment with the herbicide Diquat in potato
415 leaves. On the other hand, NO can also *S*-nitrosylate Rubisco causing reduced Rubisco
416 activity in a dose-dependent manner (Kovacs et al. 2013, Santisree et al. 2015). As possible
417 explanation for contradictory effects of NO on Rubisco, we would consider the NO donor
418 and its concentration as well as plant sensitivity, which likely vary among plant species. By
419 spraying GSNO 100 μM, our data clearly show improvements in sugarcane photosynthesis
420 through different techniques and approaches, i.e., measuring leaf gas exchange and using
421 biochemical assays.

422 GSNO also alleviated chlorophyll degradation (Fig. 1B), which is in agreement with
423 previous studies showing that exogenous application of NO may attenuate chlorophyll
424 degradation under stressful conditions (Eum et al. 2009, Wang et al. 2015). Accordingly,
425 plants sprayed with GSNO presented higher photochemical activity under water deficit as
426 compared to those sprayed with only water and also subjected to water deficit. Besides
427 showing ETR values similar to control plants during the beginning of water deficit (Fig.
428 2D), plants sprayed with GSNO presented less activity of alternative electrons sinks under
429 water deficit (Fig. 2E). Low non-photochemical quenching of chlorophyll fluorescence also
430 suggests less excessive light energy at the PSII level (Fig. 2F).

For Peer Review

1
2
3 431 Taken together, we may argue that GSNO spraying benefited plants through
4
5
6 432 improvements in leaf water status, which may be a consequence of better water balance
7
8 433 caused by increases in water supply by roots and/or reduced water loss by stomatal
9
10 434 conductance while maintaining CO_2 availability for photosynthesis. Such higher
11
12
13 435 photosynthetic rates may also be due to increases in Rubisco activity in GSNO-sprayed
14
15 436 plants with an important regulatory role at the PSII level as the main sink of ATP and
16
17 437 NADPH. While less energetic pressure at PSII level can be maintained by using of
18
19
20 438 reducing power and ATP in CO_2 assimilatory reactions under low water availability, an
21
22 439 active antioxidant system would also prevent oxidative damage induced by drought.
23
24 440 Therefore, GSNO effects on antioxidant metabolism were further investigated in both roots
25
26
27 441 and leaves of sugarcane under water deficit.
28
29 442
30
31
32 443 **GSNO improves antioxidant defense in both roots and leaves under water deficit**
33
34 444
35
36
37 445 Water deficit induced leaf $O_2^{\cdot-}$ accumulation (Fig. 5A) without a concomitant increase
38
39 446 in SOD activity (Fig. 6E) in plants without GSNO spraying. Presence of H_2O_2 in leaves of
40
41
42 447 plants under water deficit (Fig. 4B) was also found even with increases in APX activity
43
44 448 (Fig. 6C). As CAT activity was reduced and leaves presented H_2O_2 under water deficit, one
45
46
47 449 would argue that the main H_2O_2 detoxification pathway in sugarcane is through CAT
48
49 450 activity. In roots, accumulation of $O_2^{\cdot-}$ was also found under water deficit, with increases in
50
51
52 451 SOD activity and decreases in APX activity (Figs. 5A, 6D,F). Interestingly, GSNO-sprayed
53
54 452 plants did not present any increase in $O_2^{\cdot-}$ and presence of H_2O_2 in leaves (Figs. 4, 5A,B)
55
56 453 and GSNO stimulated leaf SOD activity and root CAT activity under water deficit (Figs.
57
58
59 454 4C, 6B,E).
60

For Peer Review

1
2
3
455 The increase in total SOD activity (Fig. 6E) in leaves sprayed with GSNO was
456 associated with increases in the activity of mitochondrial (*i*-Mn-SOD), chloroplastidial (*ii*-
457 Fe-SOD) and cytosolic (*iii*-Cu/Zn-SOD) isoforms (Fig. 7). As a product of SOD activity,
458 accumulation of H_2O_2 in leaves would be expected. However, evidence of H_2O_2 production
459 was not found in GSNO-sprayed plants under water deficit. It is known that the
460 detoxification of H_2O_2 can also be achieved by non-enzymatic antioxidants such as non-
461 protein thiol, ascorbate, GSH or proline. Therefore, the protective effect observed by leaf
462 GSNO spraying could be also associated with increases in GSH availability. GSNO is
463 decomposed by GSNO reductase to oxidized glutathione (GSSG), which is the substrate of
464 the glutathione reductase (GR) that regenerates GSH (Corpas et al. 2013). In fact, GSH
465 spraying improved photochemical activity in sugarcane leaf discs subjected to natural
466 drying (Silveira et al. 2016).

467 Although we have not found evidence of membrane damage under water deficit in
468 both leaves and roots (Figs. 5C,D), effects of oxidative stress on protein and DNA integrity
469 and function cannot be ruled out (Oliveira et al. 2010). Leaf and root $O_2^{\cdot -}$ concentrations
470 remained high even after the recovery period in plants previously exposed to water deficit,
471 with increased lipid peroxidation in roots (Figs. 5A,B,D). The production of ROS may
472 occur by the action of NADPH oxidase and peroxidases, causing an oxidative burst during
473 rehydration (Colville and Kranner 2010), which was also avoided in both leaves and roots
474 by spraying GSNO on leaves.

475 The changes observed in antioxidant metabolism indicate that leaf GSNO spraying
476 increased the antioxidant responses of both leaves and roots, avoiding oxidative damage
477 induced by $O_2^{\cdot -}$ and H_2O_2 under water deficit. Such responses may be related to plant water
478 status as H_2O_2 may inhibit aquaporin activity and reduce water transport (Liu et al. 2015).

For Peer Review

1
2
3
479 In fact, higher RWC and stomatal conductance in GSNO-sprayed plants were noticed (Figs. 1A, 2B). Silva et al. (2015) have already reported that sugarcane genotypes showing an active antioxidant metabolism are able to maintain root hydraulic conductance and leaf gas exchange under water deficit. Additionally, NO by itself has been reported to play an important role in aquaporin regulation by stimulating the transcription of *Os*PIP1;1, *Os*PIP1;2, *Os*PIP1;3 and *Os*PIP2;8 isoforms during rice seed germination (Liu et al. 2007). Further studies about the potential effect of NO on plant hydraulic conductance and aquaporin expression and activity should reveal another interesting interaction among this radical, antioxidant metabolism and plant water balance.

488

489 **Conclusion**

490

491 Sugarcane plants supplied with GSNO presented increases in the activity of SOD in leaves and CAT in roots, indicating higher antioxidant capacity under water deficit. Such adjustments induced by GSNO were sufficient to prevent oxidative damage in both organs and were associated with better leaf water status. As consequence, GSNO spraying alleviated the negative impact of water deficit on stomatal conductance and photosynthetic rates, with plants also showing increases in Rubisco activity under water deficit. Herein, we not only gave one step more toward the understanding of how NO may modulate plant responses to water deficit but also we provided insights about the underlying physiological processes supporting improved plant performance under stressful conditions.

500

501 **Acknowledgment**

502

For Peer Review

The authors acknowledge the financial support provided by the São Paulo Research Foundation (FAPESP, Brazil) through the BIOEN Program (Grant no. 2008/57519-2) as well as the scholarship (NMS, Grant no. 2012/19167-0) and fellowship (BBM, Grant no. 2014/13524-0) granted. LF is a European Molecular Biology Organization (EMBO) - Long Term Fellow (n°. 420/2015). The authors also acknowledge the fellowships (ABS; IS; ECM; RVR) and scholarship (FCCM) granted by the National Council for Scientific and Technological Development (CNPq, Brazil).

For Peer Review

References

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24: 1337-1344.

Azevedo RA, Alas RM, Smith RJ, Lea PJ (1998). Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiol Plant 104: 280-292.

Baker NR (2008) A probe of photosynthesis: *in vivo*. Annu Rev Plant Biol 59: 89-113.

Beligni MV, Lamattina L (2002) Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant Cell Environ 25: 737-748.

Bilger W, Schreiber U, Bock M (1995) Determination of the quantum efficiency of photo-system II and of non-photochemical quenching of chlorophyll fluorescence in the field. Oecol 102: 425-32.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

527 Broniowska KA, Diers AR, Hogg N (2013) *S*-nitrosoglutathione. Biochim Biophys Acta 528 1830: 3173–3181.

529 Cai W, Liu W, Wang WS, Fu ZW, Han TT, Lu YT (2015) Overexpression of rat neurons 530 nitric oxide synthase in rice enhances drought and salt tolerance. PLoS One 531 doi:10.1371/journal.pone.0131599.

532 Colville L, Kranner I (2010) Desiccation tolerant plants as model systems to study redox 533 regulation of protein thiols. Plant Growth Regul 62: 241-255.

534 Corpas FJ, Alché JD, Barroso JB (2013) Current over view of *S*-nitrosoglutathione (GSNO) 535 in higher plants. Front Plant Sci 4: 126-128.

536 Degl'innocenti E, Guidi L, Soldatini GF (2002) Effect of chronic O_3 fumigation on the 537 activity of some Calvin cycle enzymes in two poplar clones. Photosynthetica 40: 121-126.

538 Edwards GE, Baker NR (1993) Can CO_2 assimilation in maize leaves be predicted 539 accurately from chlorophyll fluorescence analysis? Photosynth Res 37: 89-102.

540 Eum HL, Hwang DK, Lee SK (2009) Nitric oxide reduced chlorophyll degradation in 541 broccoli (*Brassica oleracea* l. *var. italica*) florets during senescence. Food Sci Technol Int 542 15: 223-228.

543 Faoro F, Iriti M (2005) Cell death behind invisible symptoms: early diagnosis of ozone 544 injury. Biol Plant 49: 585-592.

545 Faoro F, Sant S, Iriti M, Maffi D, Appiano A (2001) Chitosan-elicited resistance to plant 546 viruses: a histochemical and cytochemical study. In: R.A.A. Muzzarelli (ed.). Chitin 547 Enzymology 57-62.

548 Farnese FS, Menezes-Silva PE, Gusman GS, Oliveira JA (2016) When bad guys become 549 good ones: the key role of reactive oxygen species and nitric oxide in the plant responses to 550 abiotic stress. Front Plant Sci 7: 471-486.

For Peer Review

551 Ferreira RR, Fornazier RF, Vitoria AP, Lea PJ, Azevedo RA (2002) Changes in antioxidant
552 enzyme activities in soybean under cadmium stress. J Plant Nutr 25: 327-342.
553 Foyer CH, Noctor G (2005) Oxidant and antioxidant signalling in plants: a re-evaluation of
554 the concept of oxidative stress in a physiological context. Plant Cell Environ 28: 1056-
555 1071.
556 Garcia-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the
557 adaptive plant responses against drought stress. Plant Physiol 126: 1196-1204.
558 Gay C, Gebicki JM (2000) A critical evaluation of the effect of sorbitol on the ferric-
559 xylenol orange hydroperoxide assay. Anal Biochem 284: 217-220.
560 Ghannoum O (2009) C4 photosynthesis and water stress. Ann Bot 103: 635-644.
561 Giannopolitis CN, Ries SK (1977) Superoxide dismutases: Occurrence in higher plants.
562 Plant Physiol 59: 309-314.
563 Hatamzadeh A, Molaahmad Nalousi A, Ghasemnezhad M, Biglouei MH (2014) The
564 potencial of nitric oxide for reducing oxidative damage induced by drought stress in two
565 turfgrass species, creeping bentgrass and tall fescue. Grass Forage Sci 70: 538-548.
566 Havir EA, Mchale NA (1987) Biochemical and development characterization of multiples
567 forms of catalase in tobacco leaves. Plant Physiol 84: 450-455.
568 Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. kinetics and
569 stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189-198.
570 Huseynova IM (2012) Photosynthetic characteristics and enzymatic antioxidant capacity of
571 leaves from wheat cultivars exposed to drought. Biochim Biophys Acta 1817: 1516-1523.
572 Jamaux I, Steinmetz A, Belhassen E (1997) Looking for molecular and physiological
573 markers of osmotic adjustment in sunflower. New Phytol 137: 117-127.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

574 Kovacs I, Lindermayr C (2013) Nitric oxide-based protein modification: formation and
575 site-specificity of protein *S*-nitrosylation. Front Plant Sci 4: 137.
576 Kuo MC, Kao CH (2003) Aluminum effects in lipid peroxidation and antioxidative enzyme
577 activities in rice leaves. Biol Plant 46: 19-152.
578 Lawlor DW (2002) Limitation to photosynthesis in water-stressed leaves: stomata vs
579 metabolism and the role of ATP. Ann Bot 62: 3235-3246.
580 Lawlor DW, Cornic G (2002) Photosynthetic carbon assimilation and associated
581 metabolism in relation to water deficits in higher plants. Plant Cell Environ 25: 275-294.
582 Liu H-Y, Yu X, Cui D-Y, Sun M-H, Sun W-N, Tang Z-C, Kwak S-S, Su W-A (2007) The
583 role of water channel proteins and nitric oxide signaling in rice seed germination. Cell Res
584 17: 638-649.
585 Liu P, Yin L, Wang S, Zhang M, Deng X, Zhang S, Tanaka K (2015) Enhanced root
586 hydraulic conductance by aquaporin regulation accounts for silicon alleviated salt-induced
587 osmotic stress in *Sorghum bicolor* L. Environ Exp Bot 111: 42-51.
588 Lopes MS, Araus JL, Van Heerden PDR, Foyer CH (2011) Enhancing drought tolerance in
589 C4 crops. J Exp Bot 62: 3135-3153.
590 Machado RS, Ribeiro RV, Marchiori PER, Machado DFSP, Machado EC, Landell MGA
591 (2009) Respostas biométricas e fisiológicas ao déficit hídrico em cana-de-açúcar em
592 diferentes fases fenológicas. PAB 44: 1575-1582.
593 Marino D, Dunand C, Puppo A, Pauly N (2012) A burst of plant NADPH oxidases. Trends
594 Plant Sci 17: 9-15.
595 Nakano Y, Asada K (1981) Hydrogen peroxide scavenged by ascorbate specific peroxidase
596 in spinach chloroplasts. Plant Cell Physiol 22: 867-880.

For Peer Review

597 Neill SJ, Desikan R, Hancock JT (2003) Nitric oxide signalling in plants. New Phytol 159:
598 11-35.
599 Oliveira M, Ahmad I, Maria VL, Pacheco M, Santos MA (2010) Antioxidant responses
600 versus DNA damage and lipid peroxidation in golden grey mullet liver: A field study at Ria
601 de Aveiro (Portugal). Arch Environ Con Tox 59: 454-463.
602 Radi R, Cassina A, Hodara R (2002) Nitric oxide and peroxynitrite interactions with
603 mitochondria. Biol Chem 383: 401-409.
604 Reid CD, Tissue DT, Fiscus EL, Strain BR (1997) Comparison of spectrophotometric and
605 radioisotopic methods for the assay of Rubisco in ozone-treated plants. Physiol Plant 101:
606 398-404.
607 Ribeiro RV, Machado RS, Machado EC, Machado DFSP, Magalhães Filho JR, Landell
608 MGA (2013) Revealing drought-resistance and productive patterns in sugarcane genotypes
609 by evaluating both physiological responses and stalk yield. Exp Agric 49: 212-224.
610 Sage RF, Sharkey TD, Seemann JR (1988) The *in-vivo* response of the ribulose-1,5-
611 bisphosphate carboxylase activation state and he pool sizes of photosynthetic metabolites to
612 elevated CO_2 in *Phaseolus vulgaris* L. Planta 174: 407-416.
613 Sales CRG, Ribeiro RV, Silveira JAG, Machado EC, Martins OM, Lagôa AMMA (2013)
614 Superoxide dismutase and ascorbate peroxidase improve the recovery of photosynthesis in
615 sugarcane plants subjected to water deficit and low substrate temperature. Plant Physiol
616 Biochem 73: 326-336.
617 Santisree P, Bhatnagar-Mathur P, Sharma KK (2015) NO to drought multifunctional role of
618 nitric oxide in plant drought: do we have all the answers? Plant Sci 239: 44-55.
619 Sarruge JR (1975) Soluções nutritivas. Summa Phytopathol 3: 231-233.

For Peer Review

620 Silva KI, Sales CRG, Marchiori PER, Silveira NM, Machado EC, Ribeiro RV (2015)
621 Short-term physiological changes in roots and leaves of sugarcane varieties exposed to
622 H_2O_2 in root medium. J Plant Physiol 177: 93-99.
623 Silva PP, Soares L, Costa JG, Viana LS, Andrade JCF, Gonçalves ER, Santos JM, Barbosa
624 GVS, Nascimento VX, Todaro AR, Riffel A, Grossi-De-Sa MF, Barbosa MHP, Sant'ana
625 AEG, Neto CER (2012) Path analysis for selection of drought tolerant sugarcane genotypes
626 through physiological components. Ind Crops Prod 37: 11-19.
627 Silveira NM, Frungillo L, Marcos FCC, Pelegrino MT, Miranda MT, Seabra AB, Salgado
628 I, Machado EC, Ribeiro RV (2016) Exogenous nitric oxide improves sugarcane growth and
629 photosynthesis under water deficit. Planta 244: 181-190.
630 Tian X, Lei Y (2006) Nitric oxide treatment alleviates drought stress in wheat seedlings.
631 Biol Plant 50: 775-778.
632 Wang Y, Luo Z, Du R (2015) Nitric oxide delays chlorophyll degradation and enhances
633 antioxidant activity in banana fruits after cold storage. Acta Physiol Plant 37: 74.
634 Wu F, Yang H, Chang Y, Cheng J, Bai S, Yin J (2012) Effects of nitric oxide on reactive
635 oxygen. Sci Hort 135: 106-111.
636 Yamamoto Y, Kobayashi Y, Devi SR, Rikiishi S, Matsumoto H (2002) Aluminum toxicity
637 is associated with mitochondrial dysfunction and the production of reactive oxygen species
638 in plant cells. Plant Physiol 128: 63-72.
639 Yun BW, Feechan A, Yin, MH, Saidi NBB, LE Bihant, YU M, Moore, JW, Kang JG,
640 Kwon E, Spoel SH, Pallas JA, Loake GJ (2011) *S*-nitrosylation of NADPH oxidase
641 regulates cell death in plant immunity. Nature 478: 264-268.

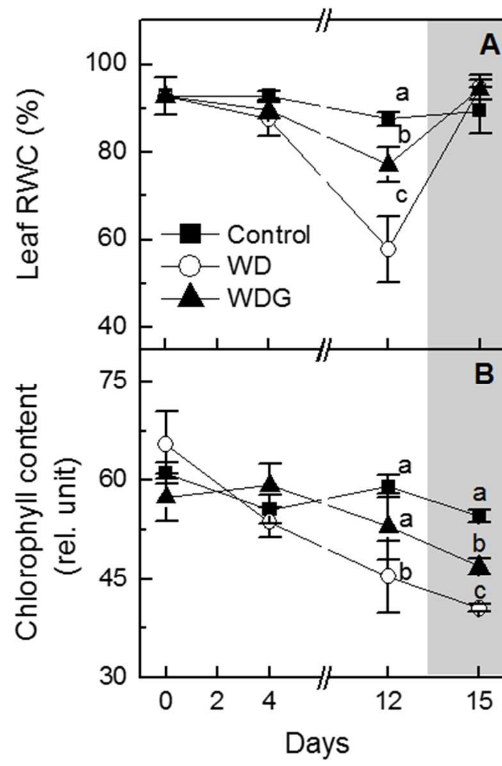
For Peer Review

642 Zhang H, Li YH, Hu LY, Wang SH, Zhang FQ, Hu KD (2008) Effects of exogenous nitric
643 oxide donor on antioxidant metabolism in wheat leaves under aluminum stress. Russ J
644 Plant Physiol 55: 469-474.
645 Zhao L, He J, Wang X, Zhang L (2008) Nitric oxide protects against polyethylene glycol-
646 induced oxidative damage in two ecotypes of reed suspension cultures. J Plant Physiol 165:
647 182-191.

For Peer Review

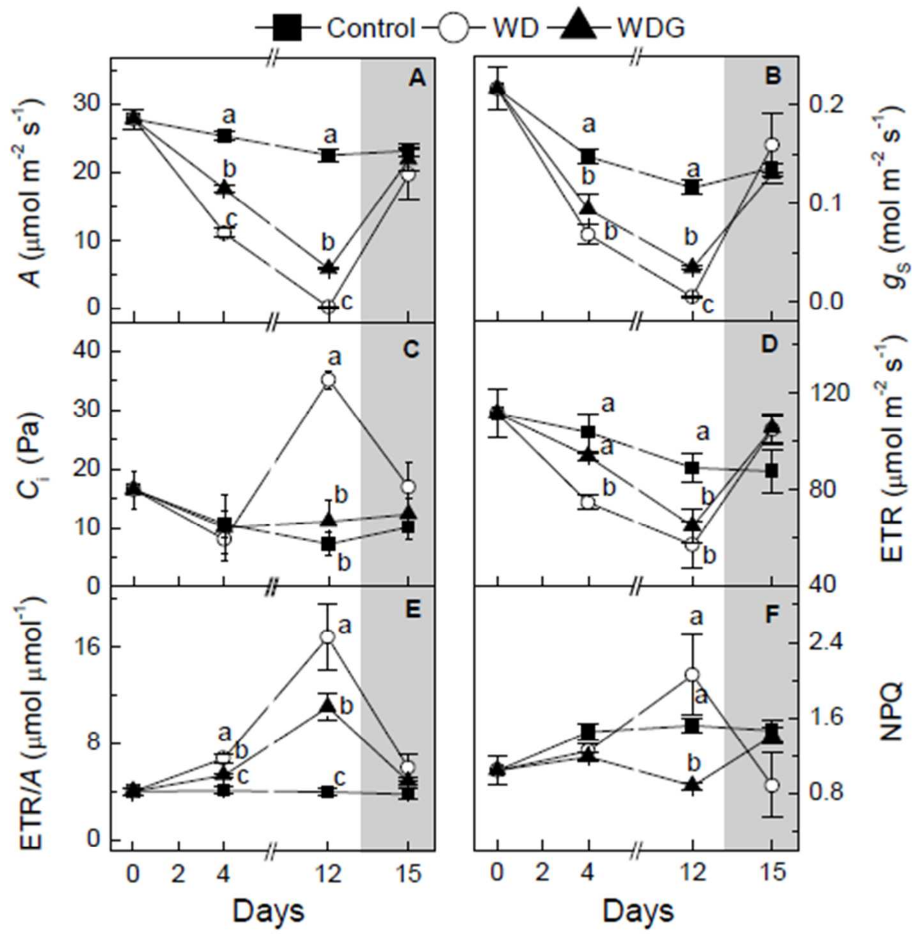
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 1



For Review

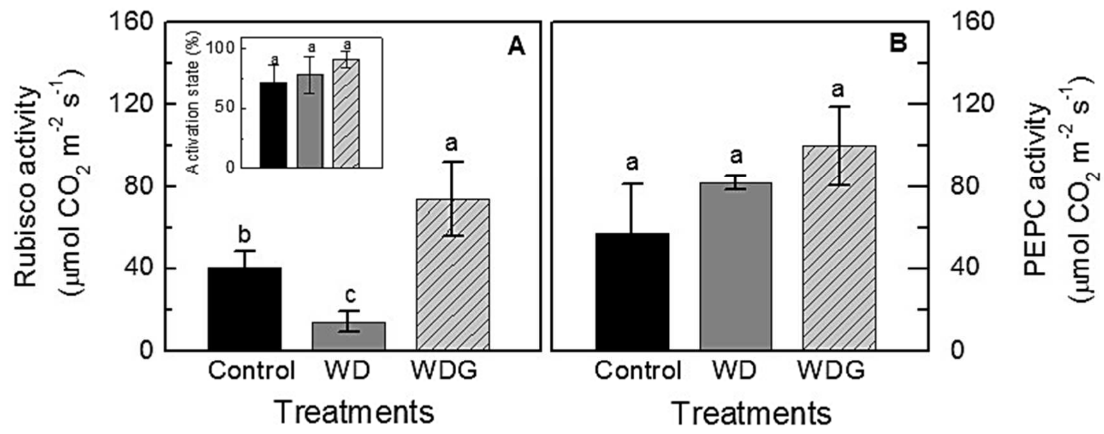
Figure 2



view

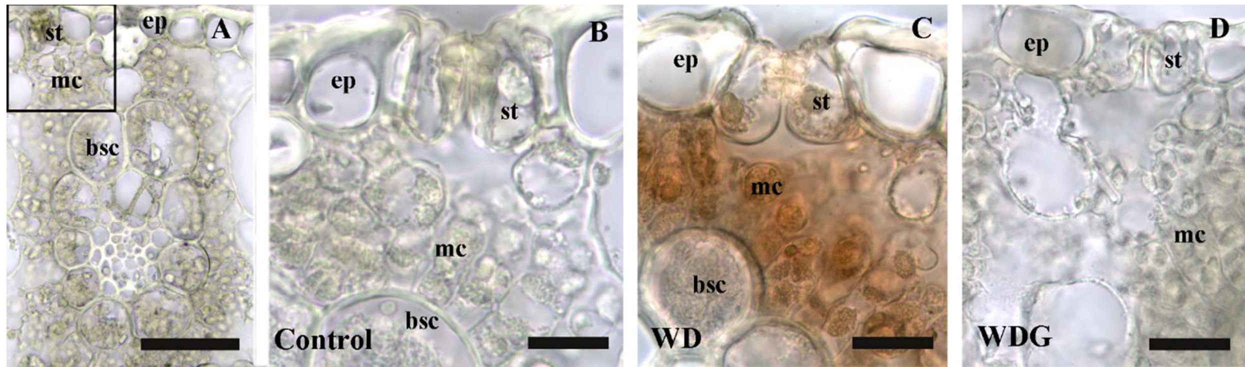
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 3



For Peer Review

1
2
3
4
5
6 Figure 4
7
8
9

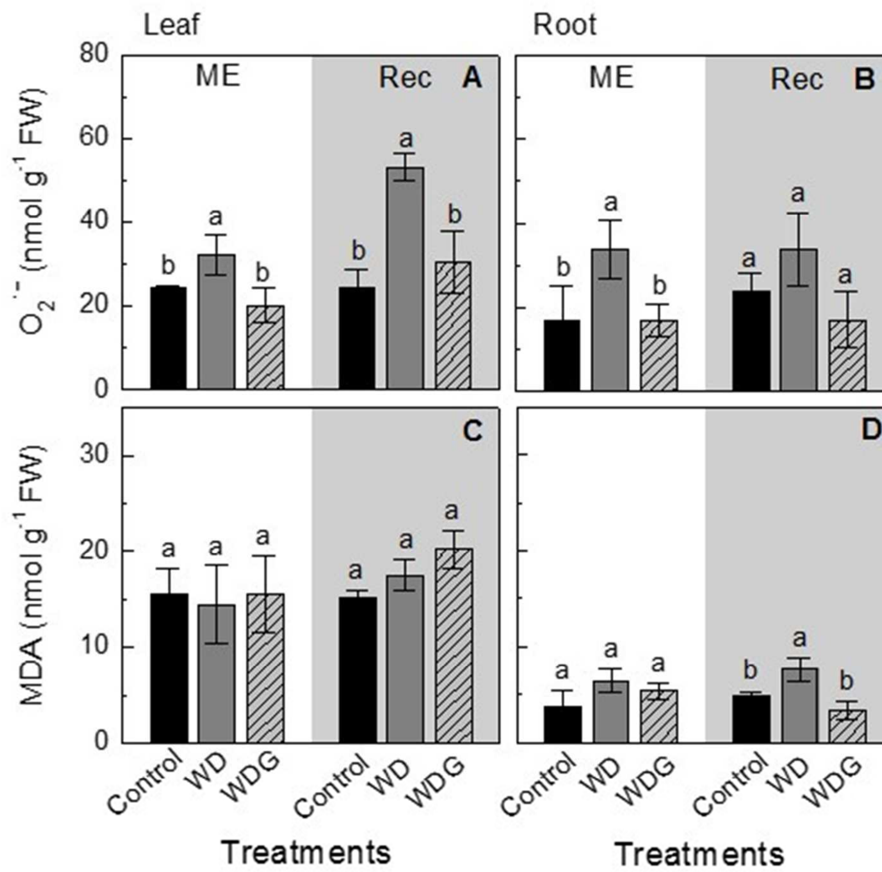


For Peer Review

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

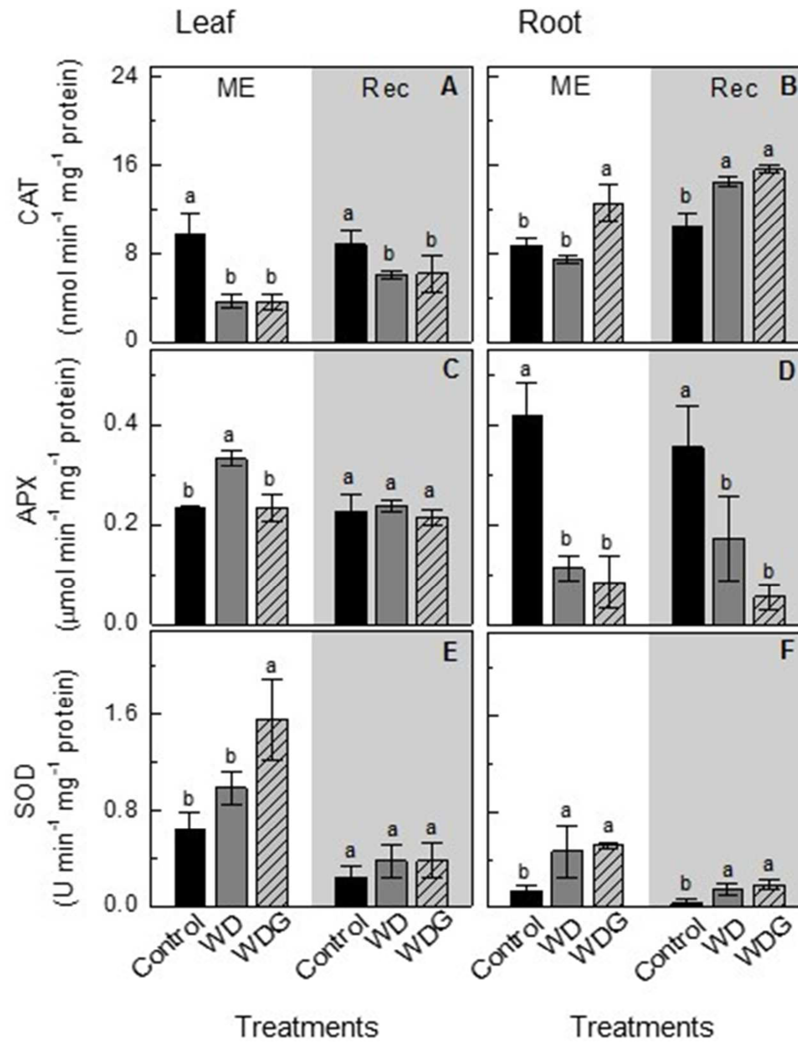
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 5



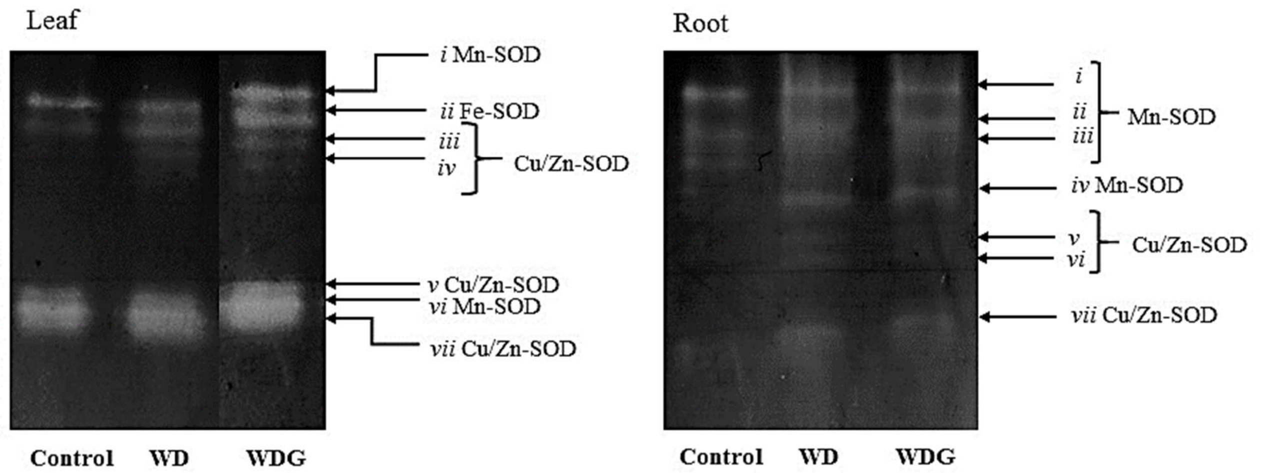
view

1
2
3
4
5
6 Figure 6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 7



Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Fig. 1. Leaf relative water content (RWC, in A) and chlorophyll content (B) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). The shaded area indicates recovery period, when plants were moved to nutrient solution used in control treatment. Each symbol represents the mean value of four replications ± standard deviation. Different lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).

Fig. 2. Leaf CO_2 assimilation (A, in A), stomatal conductance (g_S, in B), intercellular CO_2 concentration (C_i, in C), apparent electron transport rate (ETR, in D), ratio of electron transport rate and CO_2 assimilated (ETR/A, in E), and non-photochemical quenching (NPQ, in F) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). The shaded area indicates recovery period, when plants were moved to nutrient solution used in control treatment. Each symbol represents the mean value of four replications ± standard deviation. Different lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).

Fig. 3. In vitro activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, in A) and phosphoenolpyruvate carboxylase (PEPC, in B) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). Evaluations were done at the maximum water deficit. In A, Rubisco activation state is shown as insert. The data represents the mean value of three replications ± standard deviation. Different lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).

Fig. 4. Cross section of sugarcane leaves with H_2O_2 localization stained brown by DAB. A, overview of samples with no H_2O_2 accumulation, the square on the top left identify the leaf region shown in pictures B to D with mesophyll cells (mc) close to the stomata (st). B, well-hydrated (Control) samples. C, sample subjected to water deficit (WD). D, samples subjected to WD and sprayed with GSNO 100 µM (WDG). ep: epidermis, bsc: bundle-sheath cells. Bars: A = 50 µm, B-D = 20µm.

Fig. 5. Concentration of superoxide anion ($O_2^{\cdot-}$, in A and B) and malondialdehyde (MDA in C and D) in leaves (A and C) and roots (B and D) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). ME indicates maximum water deficit and Rec and shaded area indicate recovery period. The data represents the mean value of three replications ± standard deviation. Different lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).

Fig. 6. Activity of catalase (CAT, in A and B), ascorbate peroxidase (APX, C and D) and superoxide dismutase (SOD, in E and F) in leaves (A, C and E) and roots (in B, D and F) in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). ME indicates maximum water deficit and Rec and shaded area indicate recovery period. The data represents the mean value of three replications ± standard deviation. Different lowercase letters indicate statistical difference between treatments (Scott-Knott test, $P<0.05$).

Fig. 7. Superoxide dismutase isoforms by non-denaturing polyacrylamide gel electrophoresis in leaves and roots in sugarcane plants maintained well-hydrated (Control), subjected to water deficit and sprayed with water (WD) or subjected to WD and sprayed with GSNO 100 µM (WDG). Evaluations were done at the maximum water deficit. Gels are representative of three runs.

For Peer Review